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In Vitro Toxicity of Nanoparticles in BRL 3A Rat Liver Cell Lines

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FOR THE DIRECTOR

//SIGNED//

MARK M. HOFFMAN
Deputy Chief, Biosciences and Protection Division
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14. ABSTRACT

The development of nanoscience has created rapid advancement in electronics, sensors, munitions and propulsion technology. Recently, nanomaterials have received enormous attention for their potential applications in biology and medicine. However, the adverse biological affect of these nanoparticles originating from current nanotechnology has not been studied. Further comparison of these results with other nanommaterials and using other in vitro cells originating from pulmonary and skin tissues are being pursued.

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1.0. Introduction

The development of nanoscience has created rapid advancement in electronics, sensors, munitions and propulsion technology. Recently, nanomaterials have received enormous national attention for their potential applications in the manufacturing and technology sectors of the economy that includes medicinal application. Even though nanoparticles are commonly utilized for both military and industrial applications, the adverse effects of these nanoparticles originating from current nanotechnology have not been investigated. Therefore, it is necessary to assess potential adverse human health effects of nanoparticles. The major issue in evaluating the utility of these materials is assessing their potential toxicity-either due to their inherent chemical composition (e.g., chemically stable heavy metals or metal oxides) and as a consequence of their nanoscale properties. Nanomaterials, which are by definition in the 1 - 100 nanometer range, have been used to provide a fundamental understanding of phenomena and materials at the nanoscale and to create and use structures, devices and systems that have novel properties and functions, and many novel physico/chemical properties and functions are acquired at a critical length scale of matter typically under 100 nm. The manipulation of the nanoscale structures and their integration into larger material components, systems and architectures is a part of manufacturing and use of nanotechology. Although there is a wide application of nanomaterials in this modern era advancement in human history, there is a serious lack of information concerning the human health and environmental implications of manufactured nanomaterials, e.g., nanoparticles, nanotubes, nanowires, fullerene derivatives (buckyballs), and other nanoscale materials. Potentially harmful effects of nanotechnology might arise as a result of the nature of the nanoparticles themselves, the characteristics of the products made from them, or aspects of the manufacturing process involved. The large surface area, crystalline structure, and reactivity of some nanoparticles may facilitate transport in the environment or lead to adverse effects because of their interactions with cellular material. In the case of nanomaterials, stable and agglomeration size matters, and together these constraints could facilitate and exacerbate any harmful effects caused

by the composition of the material. There are limited studies conducted on inhalation studies of nanoparticles. One of the few studies was with single-wall carbon nanotubes toxicity in mice by Lam et al (2004). Their study demonstrated that carbon nanotube products induced dose-dependent epithelioid granulomas in mice and, in some cases, interstitial inflammation in the animals of the 7-day post-exposure groups. However, there are no reports on using in vitro models to evaluate toxicity screening of nanomaterials.

The use of *in vitro* models for cytotoxicity screening has increased as alternatives to whole animals testing, these methods having the advantages of decreased cost as well as increased throughput performance. Furthermore, single cell *in vitro* models provide an advantage when determining specific mechanisms of cytotoxic effects with inherently greater precision and reproducibility. At the earliest stages of toxicity screening, when the least is known about a new molecule, use of simple *in vitro* models with end points that reveal a general sense of toxicity can assist in making critical toxicity findings and allow decisions to advance to an experimental animal model and procedure as a basis for further assessing the potential risk of chemical/material exposure. In order to get a general sense of toxicity along with the maximum data in a short time, the BRL 3A immortal rat liver cell line was selected as a convenient working model system to screen nanotoxicity. The exposure to nanoparticles is likely to have potential impact on liver hepatocytes, alveolar lung cells and skin epidermal cells. These cells provide the ability to screen several nanomaterials at a low cost in a relatively rapid time frame. The toxicity end points that best represent vital biological functions of mammalian systems were selected. The toxicological tests performed were the LDH assay for viability, which measures lactate dehydrogenase leakage into media, and the MTT assay, which analyzes mitochondrial function.

The nanoparticles (Fe_3O_4 - 30, 47 nm; Ag - 15, 100 nm) were provided by Dr. J Keil from the Air Force Research Laboratory (AFRL/HEPC) to the Operational Toxicology Branch, in the Human Effectiveness Directorate (AFRL/HEBP). Before

additional tests and evaluation commenced, a general acute toxicological assessment was performed to establish a baseline toxicity measurement and selection for further studies to determine exposure risks for personnel. In deployment scenarios, there is potential concern over possible dermal or inhalation exposures to nanoparticels.

2. 0. Materials and Methods

2. 1. Chemicals: The test materials Iron Oxide (Fe_3O_4-30 nm; Fe_3O_4-47 nm) and Silver (Ag-15 nm and Ag-100 nm) were received from AFRL/HEPC. Currently limited details on the chemical properties of these materials are available. Cadmium oxide (CdO)-1000 nm from Fluka Chemicals was used as positive control and TiO_2-40nm from Altair, Nanomaterials Inc, was used as negative control to validate the toxicity studies.

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), β-nicotinamide-adenine dinucleotide-reduced (NADH), and gentamycin, were purchased from Sigma Chemical Company (St. Louis, MO). Ham's Nutrient Mixture F-12 media were obtained from Sigma Chemical Company (St. Louis, MO).

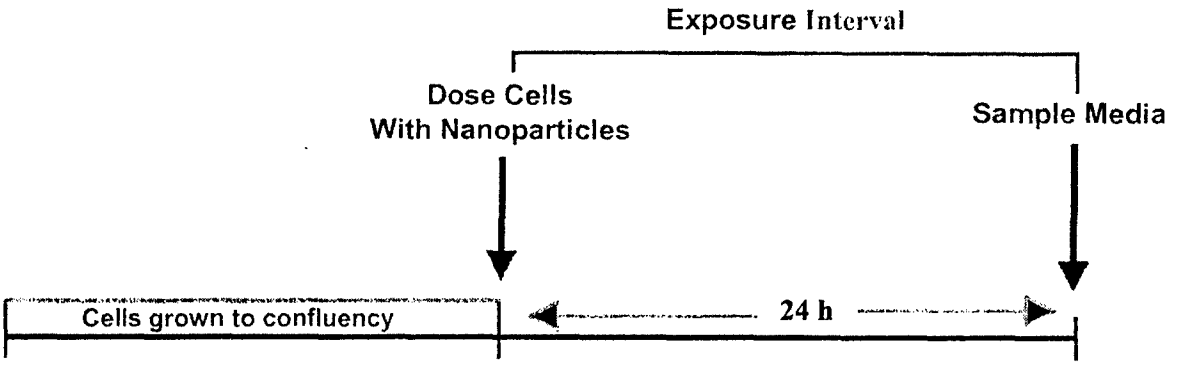
2.2. Dispersion Tests: Dispersion of nanomaterials in solution: The dispersion test was conducted in physiological phosphate buffer saline (PBS) or deionized water. Based on success of homogeneous dispersion studies using physical mixing and sonication, stock solutions were prepared either in PBS or deionized water. The stock solutions of Fe_3O_4 (30, 47 nm), TiO_2 (40 nm) and CdO (1000) nm were prepared in PBS while Ag (15, 100 nm) were prepared in deionized water. Before exposure different concentrations of nanoparticles were dissolved in Ham's Nutrient Mixture F-12 without serum.

2.3. Cell Culture. BRL 3A (ATCC, CRL-1442) immortalized rat liver cells were used between passages 25 and 30. BRL 3A cells were grown in Ham's Nutrient Mixture F-12 with 5% fetal bovine serum. Cells were plated in 6 well plates for 36-48 h

until they become confluent prior to dosing with nanoparticles. The cells were maintained in a 5% CO_2 incubator at 37°C.

2.4. Treatment and Toxicity Endpoints: Cells were plated in 6 or 24 well plates for 36-48 h until they become confluent prior to exposure. After the monolayer of cells became confluent, BRL-3A cells were treated with a range of concentrations of nanoparticles suspended in Ham's Nutrient Mixture F-12 without serum for 24 h (Figure 1). After the 24 h treatment, the following toxicity end points (LDH, MTT and external morphology) were evaluated in control and nanoparticle-exposed cells.

Figure 1



2.5.1. LDH Leakage: Membrane damage that results in LDH leakage is generally considered irreversible, therefore, LDH leakage was used as a biomarker of cellular viability. LDH leakage was assessed by measuring the activity of LDH in the cells and in the media (Moldeus et al., 1978). After treatment, the medium was removed from the culture plate and placed on ice. The plates were washed with cold phosphate buffered saline (PBS) followed by addition of 1 ml of a 0.5 % solution of Triton X-100. Plates were placed on ice for 30 min at which time the solubilized cell lysate was carefully removed and vortexed in 2 ml sample vials. Aliquots (10 μl) of the medium or cell lysate were added to 190 μl of phosphate buffer containing 0.2 mM NADH and 1.36 mM pyruvate and assayed by monitoring the rate of loss of NADH absorption at 340 nm in a SpectraMAX Plus 190 microplate reader (Molecular Devices, Sunnyvale, CA). The percent of LDH activity was then calculated by dividing the amount of activity in the medium by the total activity (medium and cell lysate). The

parallel controls were run at each time point with the cells exposed to nanoparticles-free medium. No significant difference in LDH leakage was found in controls (nanoparticle-free medium) at each time point. The OD value (340 nm) of control cells (nanoparticle-free medium at 0 h) of LDH leakage was taken as 100% and then calculated as the percentage of reduction of OD in nanoparticle-exposed cells.

2.5.2. Mitochondrial Function: Mitochondrial function was evaluated spectrophotometrically by measuring the degree of mitochondrial reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to (aqueous insoluble product) formazan by succinic dehydrogenase (Carmichael et al., 1987). Following treatment, cells were washed and incubated at 37 °C in Chee medium containing 0.05% MTT for 15 min. The medium containing MTT was removed and the colored product (formazan) was extracted from the cells in acidified isopropanol. The optical density (OD) of the extract was assayed with a SpectraMAX Plus 190 microplate reader at 570 and substracted from 530 nm (Molecular Devices, Sunnyvale, CA.).

2.5.3 Qualitative observation of external morphology of control and exposed cells by phase contrast inverted microscopy: BRL 3A cells were exposed as mentioned above at various concentrations of nanoparticles for 24 h. After completion of the exposure period, cells were washed with PBS and observed by phase contrast inverted microscopy at 100x magnification.

2.6. Statistical Evaluation: The data were expressed as mean ± standard deviation (SD) of three independent experiments. Wherever appropriate, the data were subjected to statistical analysis by one-way analysis of variance (ANOVA) followed by Dunnett's Method for multiple comparisons. A value of $p < 0.05$ was considered significant. SigmaStat for Windows version 2.03 software was used for the statistical analysis.

3.0. Results and Discussion

3.1. Physical observation on dispersion of nanoparticles

As shown in Figure 2, ferrous oxide (Fe_3O_4) and silver (Ag) dispersed in deionized water, Tris-Buffer (pH 7.4) and phosphate buffered saline (PBS) (pH 7.4). It was found that Fe_3O_4 homogenously dispersed in PBS while Ag homogenously dispersed in de-ionized water. It was noted that Ag slightly precipitated in PBS and Tris-buffer. Therefore, deionized water was selected for Ag and PBS for Fe_3O_4 as dispersion solvents to prepare stock solutions (Figures 2 A-C). From this stock solution different final concentrations were prepared in cell growth medium: Ham's media prior to exposure as shown in Figures 2 D-G. It was noted that turbidity increased with increasing concentration of nanomaterials. The turbidity intensified significantly at 250 µg/ml in all of the nanoparticles solutions. Another physical observation with Ag dispersion noted was that Ag-100 rapidly settled out of solution suspension and constant mixing was necessary before adding it to the Ham's media for exposure (Figure 2 H).

3.2. Cytotoxicity of Nanoparticles

A toxic dose range assessment study was conducted to determine the cytotoxicity resulting from nanoparticles exposure. In these studies, cells were exposed to nanoparticles at concentration ranging from 10-250 µg/ml for 24 h. At the end of the 24 h exposure period, cytotoxicity was evaluated using LDH leakage and MTT reduction as endpoints. The qualitative external morphology of control and exposed cells was assessed by phase contrast inverted microscopy.

Membrane damage that results in LDH leakage is generally considered irreversible; therefore, LDH leakage was selected as a biomarker for cellular viability (Hussain et al., 2001). The results for LDH leakage indicate that Fe_3O_4 nanoparticles exposure did not produce cytotoxicity up to the concentration of 100 µg/ml (Figure 3 A), but it produced a significant effect at 250 µg/ml. This was the same concentration as the negative control (TiO_2) suggesting a nonspecific response at this high concentration (Figure 9A). The Ag exposure exhibited a significant cytotoxicity at 5-50

μg/ml (Figure 3 B). In these LDH leakage studies, there was no significant divergence when comparing the toxic effects between different sizes of Fe_3O_4 nanoparticles (30 nm versus 47 nm). However, it was noted that there is a statistically significant difference between 100 and 30 nm of silver, where the 100 nm particles showed higher toxicity at higher dose.

Mitochondria are vulnerable targets for toxic injury by a variety of compounds because of their crucial role in maintaining cellular structure and function via aerobic ATP production (Hussain et al., 2002). Therefore, the MTT assay that determines mitochondrial function was selected as a biomarker to assess if the nanoparticles affect mitochondrial function. The results of MTT assays showed that the Fe_3O_4 nanoparticles did not produce significant increase in cytotoxicity at the doses tested (Figure 4A) except at 250 μg/ml. The toxicity at 250 μg/ml seems to be not chemical composition specific as the toxicity appears in negative control (TiO_2) at the same concentration (250 μg/ml). There was a slight increase in mitochondrial function at the 10 μg/ml doses of nanoparticles, but these were not statistical significant (Figure 4A). There was relatively no change in MTT function indicating that the mitochondrial function was not altered at these doses. In contrast, Ag exposure exhibited a significant cytotoxicity from 5-50 μg/ml (Figure 4B). At 50 μg/ml there was about 90% reduction in mitochondrial function. As assessed by MTT assay, silver nanoparticles were more toxic than Fe_3O_4 nanoparticles. In addition, there was no difference in toxicity when different sizes of Fe_3O_4 and Ag nanoparticles were evaluated.

Figure 5 shows the general external morphology of control cells and nanoparticle-exposed cells. The Fe_3O_4 nanoparticles appeared to be associated with cells exhibiting a brownish color when observed under the microscope. These results demonstrated no change in the shape of the cells when exposed to iron oxide (Figures 5 & 6). The cells exposed to silver showed a drastic change at higher concentrations (Fig 7 & 8). At the low dose (10 μg/ml), the cells appear similar to control cells with reddish particles most likely associated with the cell membranes. With increasing doses of Ag nanoparticles, the BRL 3A cells started to shrink and became irregular in

shape (Figures 7 & 8). These microscopic studies indicate that not all nanoparticles particles were accumulated into the cells, rather some associated with membranes.

Validation Results with Reference Chemicals

In order to validate the assay system, we compared results of nanoparticles with other known positive and negative controls such as cadmium oxide (CdO) and , titanium dioxide (TiO_2-40nm). The EC_{50} values of these compounds are shown in Table 1. The TiO_2 did not exhibit toxicity up to 100 µg/ml in the BRL 3A liver cell lines (Figure 9 A & B). However, the positive control (CdO) displayed dose dependent toxicity with 80% cell death at 10 µg/ml (Figure 9A) as determined by MTT reduction and LDH assay.

In conclusion based on our preliminary toxicity assessment Ag nanoparticles are more toxic than Fe_3O_4. These results provide an early indication of potential toxicity concern of silver nanoparticles and may require further assessment to determine their biological interaction and potential adverse effects.

Table 1: Calculated EC_{50} values of reference chemicals

Chemical	MTT EC_{50} µg/ml	LDH EC_{50} µg/ml
CdO-1000 nm	0.83 ± 0.027	0.75 ± 0.052
Ti Nano-40 nm	>250	>250
Ag-100 nm	19 ± 5.2	24 ± 9.25
Ag-15 nm	24 ± 7.25	50 ± 10.25
Fe3O4-30 nm	>250	>250
Fe3O4-47 nm	>250	>250

References:

Carmichael, J., DeGraff, W.G., Gazdar, A.F., Minna, J.D., and Mitchell, J.B. (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. *Cancer Res.* **47,** 936-942.

Hussain, S.M. and Frazier, J.M (2001). *In vitro* assessment of high energy chemicals in rat hepatocytes. *Sci. Total Environ.* **274,** 151-160.

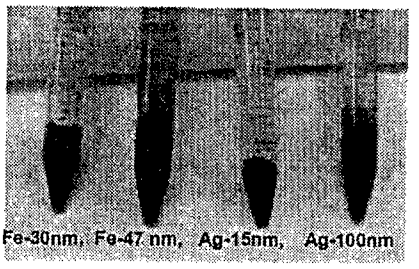
Hussain, S.M. and Frazier, J.M (2002) Cellular toxicity of hydrazine in primary hepatocytes. *Toxicol Sciences 69, 424-432.*

Moldeus, P., Hogberg, J. and Orrenius, S. (1978) Isolation and use of liver cells. *Meth. Enzymol.* **52,** 60-72.

Lam CW, James JT, McCluskey R, and Hunter RL (2004) Pulmonary Toxicity of Single-Wall Carbon Nanotubes in Mice 7 and 90 Days After Intratracheal Instillation Toxicological Sciences **77,** 126–134.

Figure 2

A.



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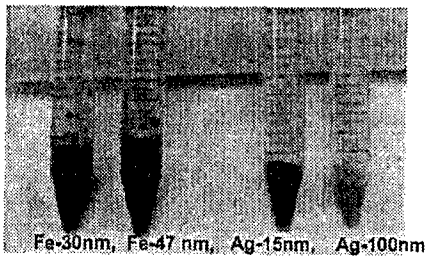

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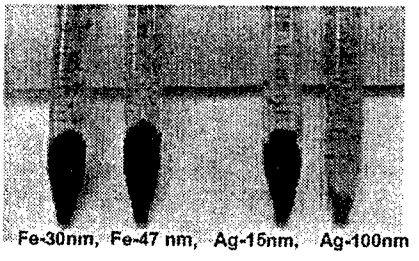

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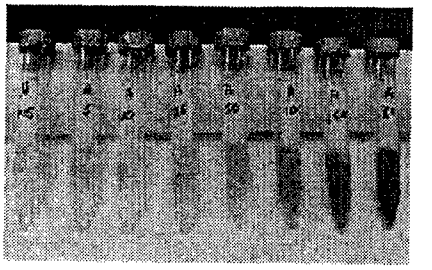
D



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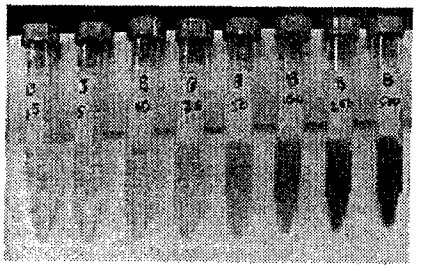

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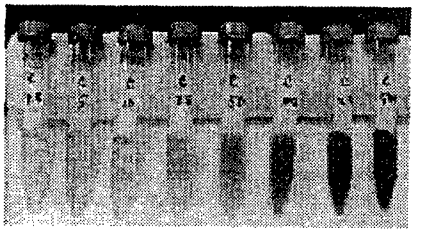

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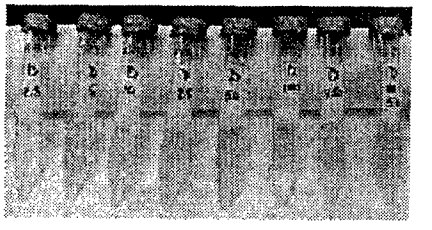
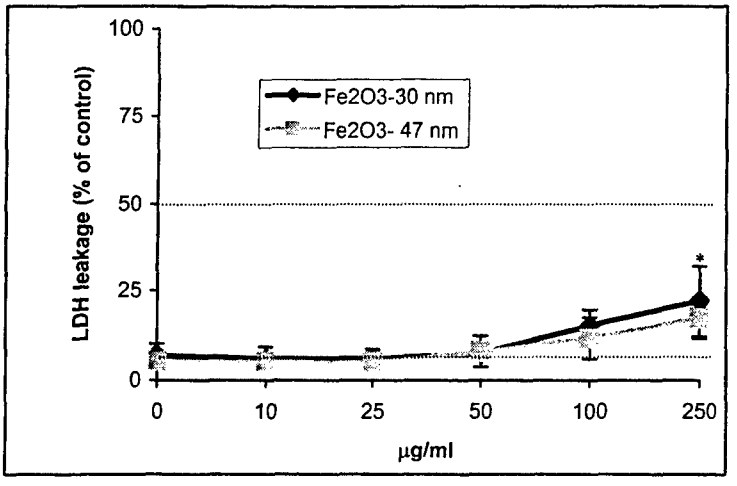


Figure 3 A


100
75
50
25
0
LDH leakage (% of control)
Fe2O3-30 nm
Fe2O3- 47 nm
0
10
25
50
100
250
µg/ml

3 B

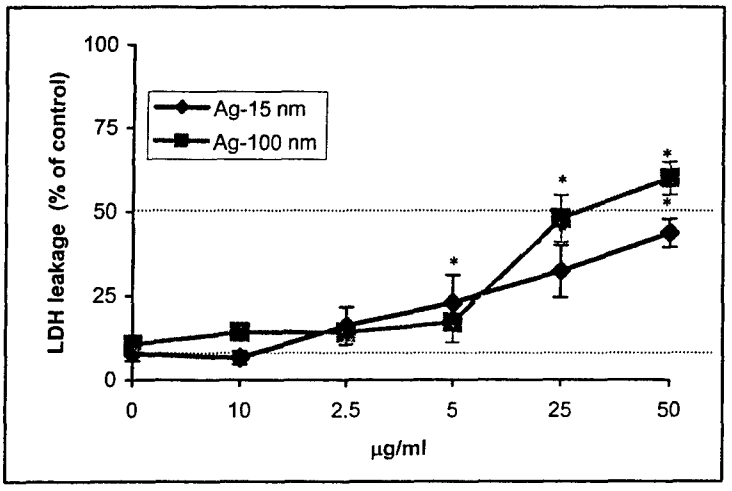
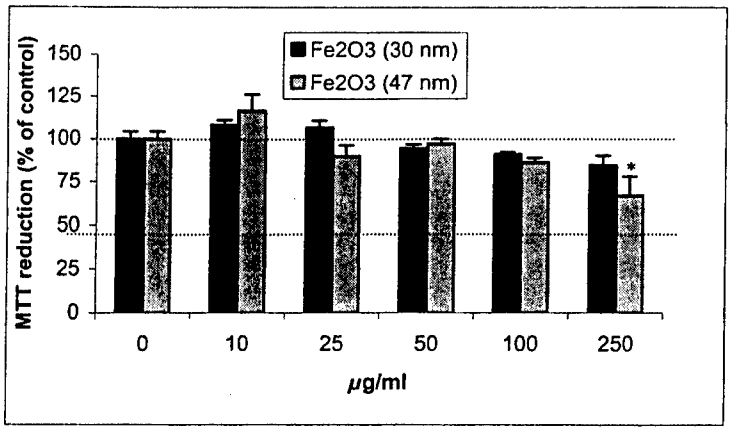

100
75
50
25
0
LDH leakage (% of control)
Ag-15 nm
Ag-100 nm
0
10
2.5
5
25
50
µg/ml

Figure 4 A


Fe2O3 (30 nm)
Fe2O3 (47 nm)
MTT reduction (% of control)
150
125
100
75
50
25
0
0
10
25
50
100
250
µg/ml
*

4 B

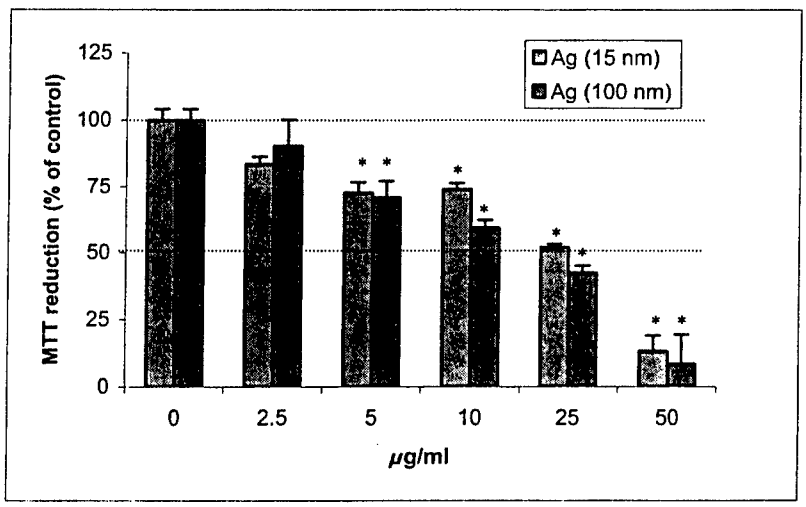

Ag (15 nm)
Ag (100 nm)
MTT reduction (% of control)
125
100
75
50
25
0
0
2.5
5
10
25
50
µg/ml
* * * * * * * * * * * *

Figure 5. Qualitative observation of external morphology of control and Fe_3O_4-30 nm exposed cells by phase contrast inverted microscopy. A Control; B 10 µg/ml; C 50 µg/ml; D 100 µg/ml.

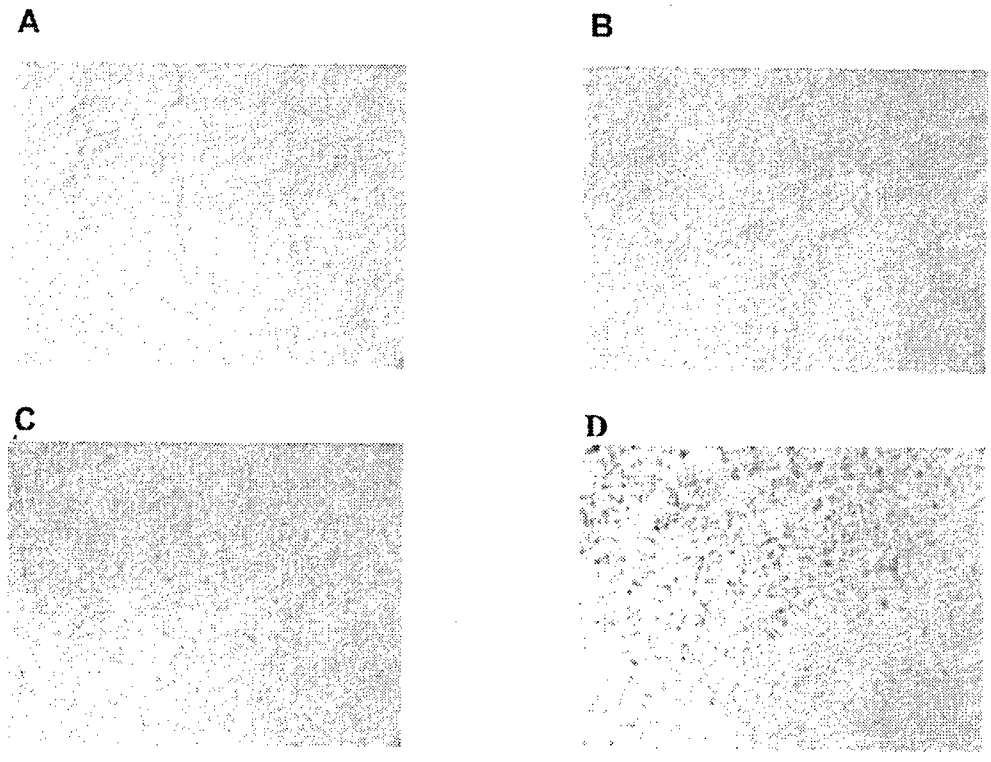


Figure 6. Qualitative observation of external morphology of control and Fe_3O_4-47 nm exposed cells by phase contrast inverted microscopy. A Control; B 10 µg/ml; C 50 µg/ml; D 100 µg/ml.

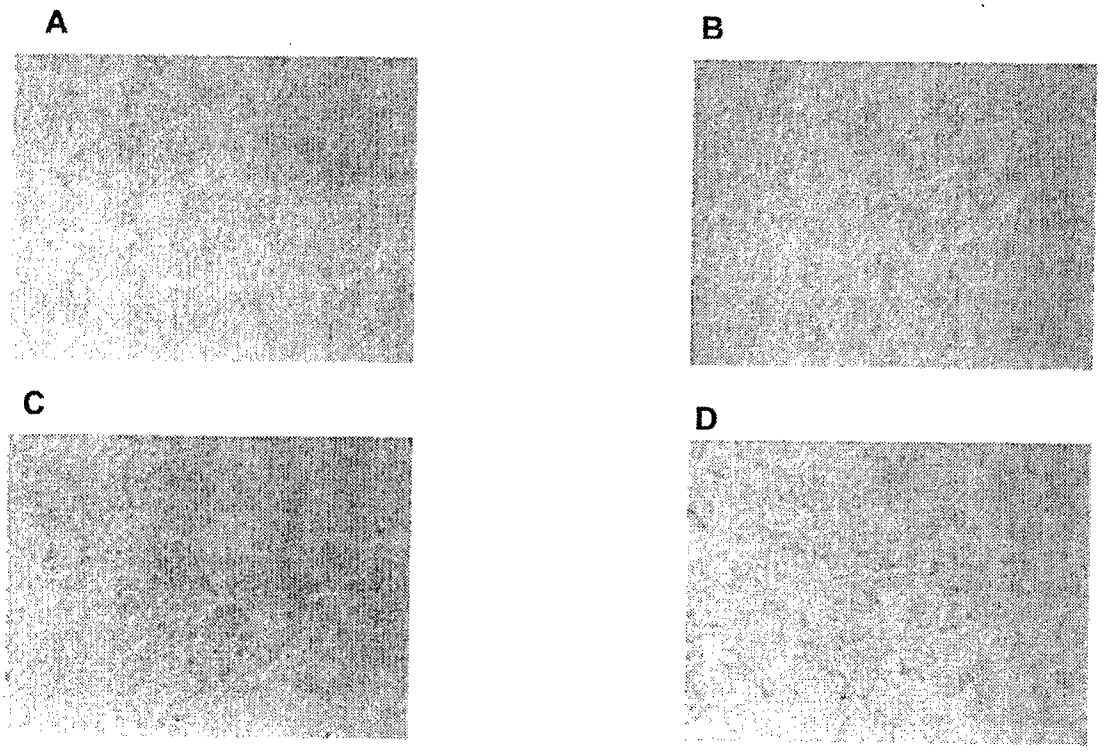


Figure 7. Qualitative observation of external morphology of control and Ag-15 nm exposed cells by phase contrast inverted microscopy. A Control; B 10 µg/ml; C 50 µg/ml; D 100 µg/ml.

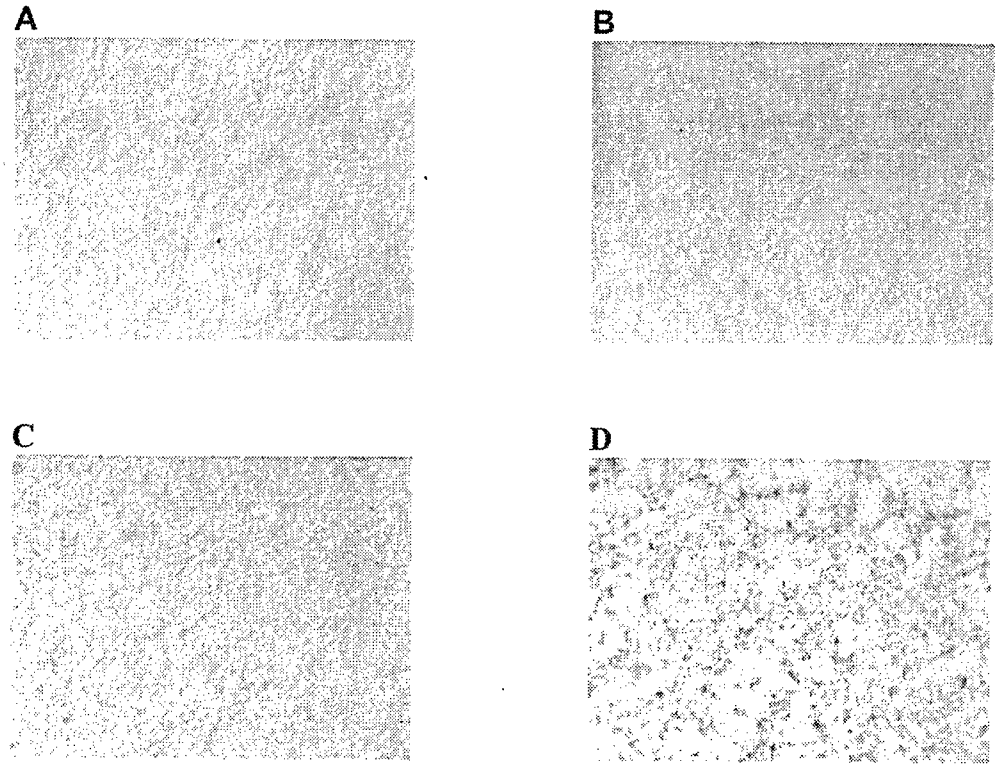
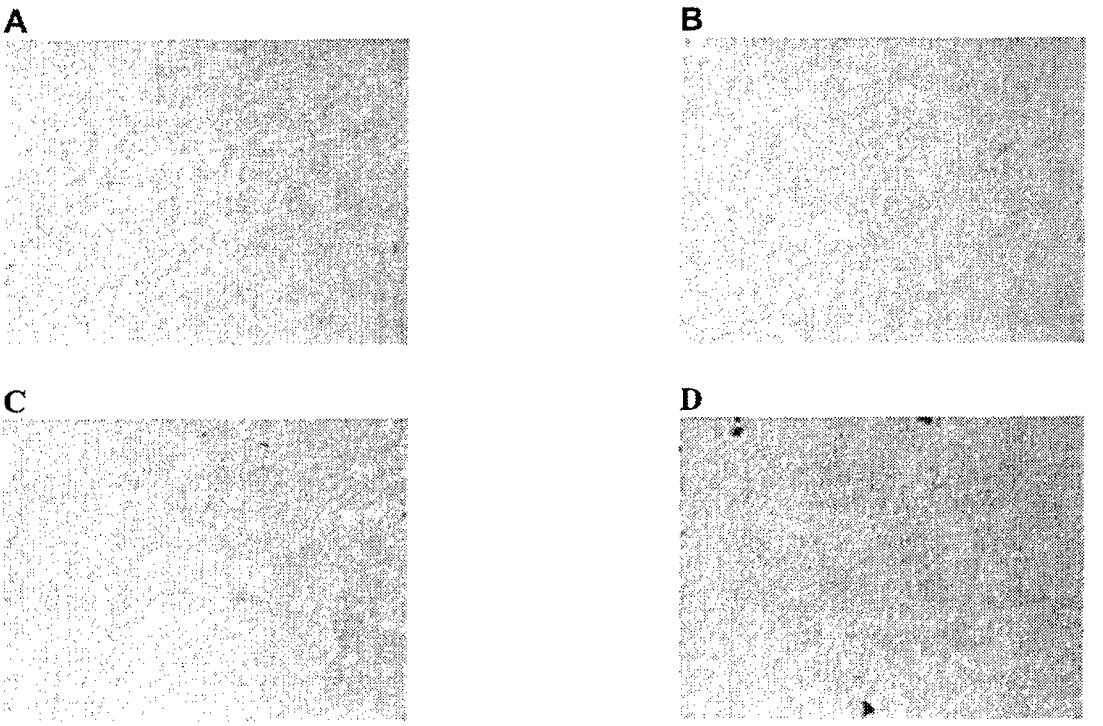
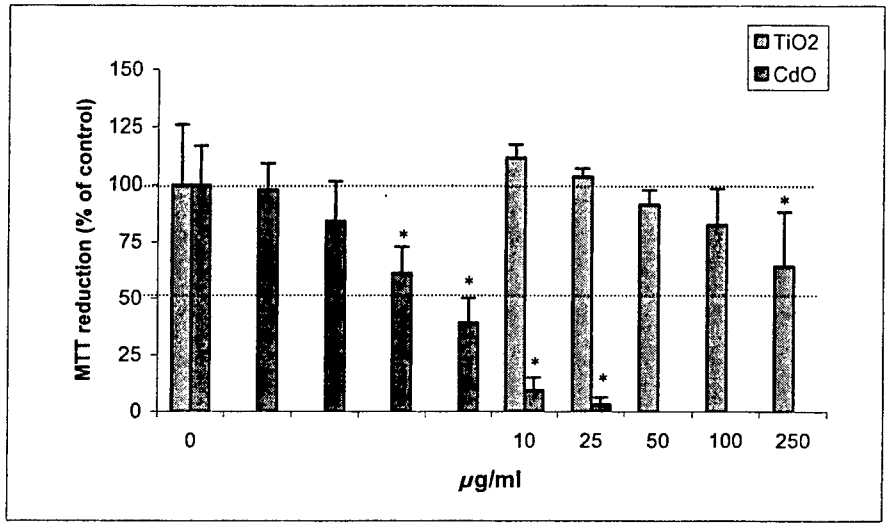


Figure 8. Qualitative observation of external morphology of control and Ag-100 nm exposed cells by phase contrast inverted microscopy. A Control; B 5 µg/ml; C 10 µg/ml; D 50 µg/ml.



9 A


TiO2
CdO
MTT reduction (% of control)
150
125
100
75
50
25
0
0
10
25
50
100
250
µg/ml

9 B

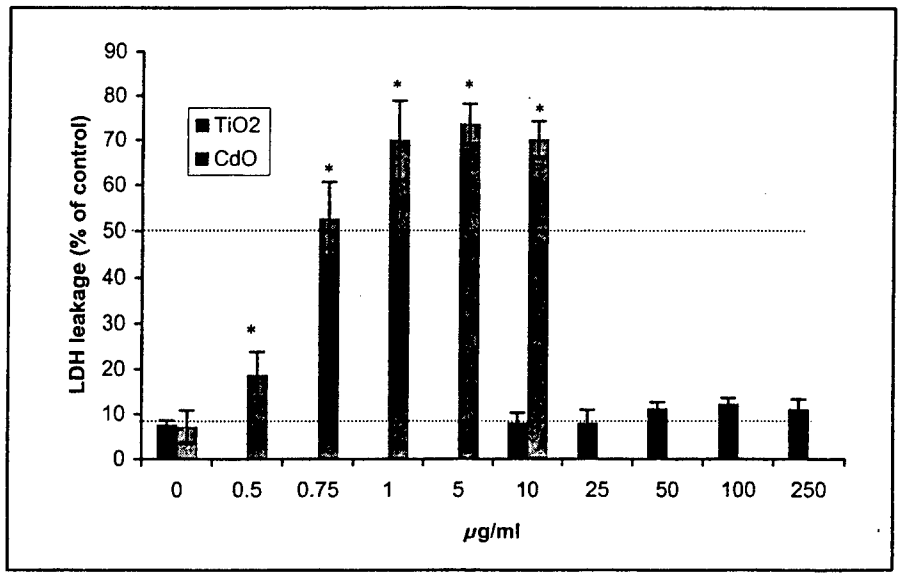

TiO2
CdO
LDH leakage (% of control)
90
80
70
60
50
40
30
20
10
0
0
0.5
0.75
1
5
10
25
50
100
250
µg/ml

Figure 2 A. Nanoparticles dispersed in dH2O

Figure 2 B. Nanoparticles dispersed in Tris-buffer pH 7.4

Figure 2 C. Nanoparticles dispersed in PBS pH 7.4

Figure 2 D. Different concentrations of Iron Oxide - 30 nm (μg/ml) in Hams media before exposure to BRL cells

Figure 2 E. Different concentrations of Iron Oxide - 47 nm (μg/ml) in Hams media before exposure to BRL cells

Figure 2 F. Different concentrations of Silver -15 nm (μg/ml) in Hams media before exposure to BRL cells

Figure 2 G. Different concentrations of Silver-100 nm (μg/ml) in Hams media before exposure to BRL cells

Figure 2 H. Silver 100 nm settled out of suspension rapidly.